Tetragenococcus koreensis sp. nov., a novel rhamnolipid-producing bacterium

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A Gram-positive, non-motile, non-spore-forming coccus (strain JS^T) was isolated from kimchi (a traditional Korean food) and investigated using a polyphasic taxonomic approach. The 16S rRNA gene sequence similarity between strain JS^T and its closest relative, *Tetragenococcus halophilus* IAM 1676^T, was 98.1 %. The level of DNA–DNA relatedness between the two strains was 9.7 %. Strain JS^T had a DNA G + C content of 38.3 % and a cellular fatty acid profile containing 16 : 0, 18 : 1 and cyclo fatty acids. Phylogenetic data and genomic and phenotypic features demonstrated that strain JS^T represents a novel species, for which the name *Tetragenococcus koreensis* sp. nov. is proposed. The type strain is JS^T (= KCTC 3924^T = DSM 16501^T = LMG 22864^T).

The genus *Tetragenococcus* is classified within the family 'Enterococcaceae', which belongs to the order 'Lactobacillales' in the class 'Bacilli' (Garrity & Holt, 2001). The genus was created by Collins *et al.* (1990) after reclassification of the halophilic lactic acid bacterium [*Pediococcus*] *halophilus* as *Tetragenococcus halophilus*. At present, the genus comprises three species: *T. halophilus*, *Tetragenococcus muriaticus* (Satomi *et al.*, 1997) and *Tetragenococcus solitarius* (Ennahar & Cai, 2005).

Kimchi is a common food in Korea. It is prepared with various kinds of vegetables, spices and other ingredients, and becomes palatable through ageing under anaerobic conditions. Kimchi fermentation is initiated by various micro-organisms present in the raw materials, but is gradually dominated by lactic acid bacteria.

Strain JS^T was isolated from a traditional Korean food, kimchi, by direct plating on glucose yeast peptone (GYP)/sodium acetate/mineral salts agar at pH 7.0 (medium 243; BCCM). After 7 days incubation on GYP/sodium acetate/mineral salts agar, colonies were smooth, low convex, circular, opaque, matt and white in colour. Strain JS^T grew at

15–30 °C but not at, or above, 4 °C or at temperatures above 42 °C. The strain grew optimally at pH 9. Optimal growth was obtained in 2–5 % (w/v) NaCl. Strain JS^T did not grow under aerobic conditions on R2A agar (Difco), nutrient agar, MacConkey agar (Difco) or trypticase soy agar (Difco) at 30 °C. For maintenance of the culture and further analyses, strain JS^T was grown on MRS agar (Difco) at 30 °C, unless specified otherwise.

Cell morphology and motility were observed under a Nikon Optiphot 111675 light microscope (1000 × magnification). Cell morphology was also examined by using scanning electron microscopy. The procedures used for sample fixation and preparation were as described by Chung & Hancock (2000), with some modifications. Cells were non-spore-forming, non-motile cocci occurring singly or in pairs. The cell diameter was less than 1 µm (see the Supplementary Figure in IJSEM Online) on GYP/sodium acetate/mineral salts agar. The Gram reaction, determined by the non-staining method described by Buck (1982), was positive. Oxidase activity was tested using the oxidation of 1 % *p*-aminodimethylaniline oxalate. Catalase activity was determined using bubble production with 3 % (v/v) hydrogen peroxide solution. Strain JS^T showed no catalase or oxidase activities.

For 16S rRNA gene sequencing, cell biomass was obtained from cultures grown in MRS broth (Difco) on a horizontal

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain JS^T is AY690334.

A scanning electron micrograph of cells of strain JS^T is available as supplementary material in IJSEM Online.

Acid production from carbohydrates was determined as described by Hugh & Leifson (1953). The API 20NE, API 32GN, API 50 CH and API 20 Strep microtest systems were used according to the recommendations of the manufacturer (bioMérieux). Strain JS^T did not reduce nitrate to nitrite or nitrogen gas. Strain JS^T had β -glucosidase and β -galactosidase activity but protein was not hydrolysed. The differential phenotypic properties of strain JS^T and other type strains of species in the genus *Tetragenococcus* are summarized in Table 1.

The peptidoglycan structure of the cell wall was determined as described by Schleifer (1985) and Schleifer & Kandler (1972), with the modification that TLC on cellulose was used instead of paper chromatography. The total hydrolysate of the peptidoglycan contained the amino acids alanine and glutamic acid plus smaller amounts of lysine and aspartic acid. In addition, the peptide L-Lys-D-Asp was detected; it is stable under the conditions used for total hydrolysis (100 °C, 4 M HCl, 16 h). Partial hydrolysis (100 °C, 4 M HCl, 45 min) resulted in the peptides L-Ala-D-Glu and L-Lys-D-Ala. From these data it was concluded that strain J5^T possessed peptidoglycan type A4 α L-Lys-D-Asp (type A11.31 according to <http://www.dsmz.de/species/murein.htm>).

For DNA–DNA hybridizations, DNA was extracted as

Table 1. Phenotypic characteristics that differentiate strain JS^T from other members of the genus *Tetragenococcus*

Taxa: 1, *T. halophilus* IAM 1676^T; 2, *T. muriaticus* JCM 10006^T; 3, *T. solitarius* DSM 5634^T; 4, strain JS^T. All strains were Gram-positive cocci, non-motile, non-pigmented, catalase-negative and halophilic. All strains hydrolysed aesculin and had urease activity. All strains were positive for fermentation of *N*-acetylglucosamine, D-glucose, D-fructose and salicin. All strains were negative for fermentation of L-fucose, 5-ketogluconate, lactose, D-lyxose, D-melibiose, methyl β -D-xyloside, D-raffinose, L-rhamnose, L-xylose, glycogen, starch, adonitol, L-arabitol, erythritol, glycerol, sorbitol and inulin. +, Positive reaction; –, negative reaction; W, weakly positive reaction; ND, no data.

Characteristic	1	2	3	4
Morphology	Spherical, pairs	Tetrads, pairs	Ovoid	Cocci
Peptidoglycan	Lys–D–Asp	Lys–D–Asp	ND	Lys–D–Asp
Oxidase	–	–	ND	–
Growth at 45 °C	–	–	+	–
Optimum NaCl conc. (% w/v)	5–10	7–10	ND	2–5
NaCl range (% w/v)		1–25	6–50	0–8
Nitrate reduction	–	–	ND	–
Lactic acid production (homofermentative)	+	+	ND	+
Growth on MRS	–	–	ND	+
Fermentation of sugars				
L-Arabinose	+	–	–	–
D-Cellobiose	+	–	+	–
D-Galactose	+	–	+	+
2-Ketogluconate	W	–	–	–
Maltose	+	–	+	+
D-Mannose	+	+	+	+
Melezitose	–	–	+	+
D-Ribose	+	+	–	+
Sorbose	–	–	–	–
D-Sucrose	+	–	+	+
D-Tagatose	–	–	+	–
D-Trehalose	+	–	+	+
Turanose	–	–	+	+
D-Xylose	+	–	–	+
Methyl α -D-glucoside	–	–	–	+
Methyl α -D-mannoside	–	–	–	+
D-Arabitol	–	–	+	+
Dulcitol	–	–	+	–
Mannitol	–	+	+	+
Xylitol	–	–	–	+
Amygdalin	+	–	+	–
Arbutin	+	–	+	+
Gluconate	+	–	+	+
DNA G + C content (mol%)	34–36	36.5	38.0	38.3

indicated above. Hybridizations were performed fluorometrically by the method of Ezaki *et al.* (1989), using photobiotin-labelled DNA probes and microdilution wells, with five replications for each sample. The highest and lowest values obtained for each sample were excluded; the remaining three values were used to calculate similarity values. The DNA relatedness values quoted are the means of these three values. Strain JS^T exhibited low levels of DNA–DNA hybridization: 9.7 % with *T. halophilus* IAM 1676^T, 61.0 % with *T. solitarius* DSM 5634^T and 39.0 % with *T. muriaticus* JCM 10006^T.

Lactic acid production was analysed by using HPLC (Acme

HPLC; Younglin Instrument Co.). The HPLC analysis was performed with an HPLC system (processor, pump model SP930D, UV detector model UV 730D, column oven, auto-sampler) from Younglin Instrument Co. This was equipped with an Aminex HPX-87H organic acid analysis column from Bio-Rad. A 20 μ l aliquot of the 10^{–3} dilution of strain JS^T culture broth was injected; 0.0035 M H₂SO₄ solution was used as eluent, at 30 °C and at a flow rate of 0.7 ml min^{–1}. The lactic acid in the culture broth was detected using a UV detector (210 nm) (Younglin Instrument Co.) and compared with a standard lactic acid from Aldrich, USA. Strain JS^T produced lactic acid homofermentatively.

Table 2. Cellular fatty acid profiles of strains JS^T and other closely related species in the genus *Tetragenococcus*

Taxa: 1, *T. halophilus* IAM 1676^T; 2, *T. muriaticus* JCM 10006^T; 3, *T. solitarius* DSM 5634^T; 4, strain JS^T. For unsaturated fatty acids, the position of the double bond is located by counting from the methyl (ω) end of the carbon chain; *cis* and *trans* isomers are indicated by the suffixes *c* and *t*, respectively.

Fatty acid	1	2	3	4
Saturated fatty acids				
12:0	–	0.9	2.7	–
14:0	8.8	8.0	18.5	7.4
16:0	28.9	21.9	23.3	22.4
17:0 cyclo	1.5	–	2.8	–
18:0	–	1.1	2.7	–
19:0 cyclo ω 8 <i>c</i>	14.6	6.3	12.0	26.8
19:0 cyclo ω 10 <i>c</i>	–	–	3.7	–
Unsaturated fatty acids				
16:1 ω 7 <i>c</i>	16.1	18.9	13.4	9.6
18:1 ω 7 <i>c</i> /9 <i>t</i> /12 <i>t</i>	25.4	36.6	11.9	33.8
18:1 ω 9 <i>c</i>	2.1	2.0	4.0	–
18:2 ω 6,9 <i>c</i>	–	1.3	2.8	–
Branched fatty acids				
15:0 iso 2-OH	2.5	2.0	2.5	–
16:0 2-OH	–	1.0	–	–

For the rhamnolipid production assay, HPLC was carried out using a Waters Altantis dC₁₈ reversed-phase column (150 × 3.9 mm, 5 μ m). An aliquot (20 μ l) of the strain JS^T culture broth was injected; 100 % water was used as eluent at 30 °C with a flow rate of 0.6 ml min^{−1}. Products were detected using a UV detector (204 nm), then compared with a standard rhamnolipid biosurfactant from the Korea Research Institute of Bioscience and Biotechnology (Daejeon, Republic of Korea). The surface tension of the cell-free culture broth was determined with a Thermo Cahn tensiometer with the WinDCA32 software suite (Cahn Instruments) according to the De Nöuy ring method (Zajic & Seffens, 1984). To estimate the emulsification activity, 5 ml n-hexadecane was added to 5 ml cell-free culture broth in graduated test tubes. These tubes were then vortexed for 2 min and allowed to stand at 30 °C. The emulsion stability was measured after incubation for 24 h. The emulsification index (E₂₄ %) was calculated by dividing the height of the emulsion layer by the total height of the mixture and multiplying the quotient by 100. The use of biosurfactants generally results in foaming, a decrease in surface tension, and emulsification of hydrocarbon substrates. The growth of strain JS^T on 2–5 % (w/v) NaCl on GYP/sodium acetate/mineral salts agar for 7 days lowered the surface tension to 34.9 dyne cm^{−1}, and the emulsifying activity was 61.5 %. It has been reported that rhamnolipids were produced by *Pseudomonas aeruginosa* (Rahman *et al.*, 2002) from various substrates, including industrial waste (Haba *et al.*, 2000). They are as diverse, in terms of chemical structure and properties, as the producer

micro-organisms (Kosaric, 1987). Biosurfactants are biodegradable, less toxic, less allergenic and therefore less detrimental to the environment.

The overall results of the present study allowed us to assign strain JS^T to a novel species, for which we propose the name *Tetragenococcus koreensis* sp. nov.

Description of *Tetragenococcus koreensis* sp. nov.

Tetragenococcus koreensis (ko.re.en'sis. N.L. masc. adj. *koreensis* pertaining to Korea, the origin of the sample of the traditional food, kimchi, from which the type strain was isolated).

Cells are non-motile, non-spore-forming cocci (approx. 1 μ m in length). Gram-positive, catalase-negative, oxidase-negative. Grows on trypticase soy agar at 30 °C aerobically and in an anaerobic jar, but does not grow on R2A agar, nutrient agar or MacConkey agar at 30 °C under either aerobic or anaerobic conditions. Fatty acid profile largely composed of C_{18:1} ω 7*c* (33.8 %), C_{19:0} cyclo ω 8*c* (26.8 %) and C_{16:0} (22.4 %). Growth occurs at 15–30 °C on GYP/sodium acetate/mineral salts agar but not at temperatures of 4 °C or above 37 °C. Optimum growth temperature is 30 °C. Optimal pH is 9.0. Growth occurs at NaCl concentrations in the range 0–8 % (w/v). The NaCl concentration for best growth is 2–5 % (w/v). Facultatively aerobic. Demonstrates homofermentative production of lactic acid. The peptidoglycan type is A4 α L-Lys–D-Asp. Nitrate is not reduced to nitrite or nitrogen gas. The G + C content of the DNA is 38.3 mol%. β -Glucosidase- and β -galactosidase-positive, but protein is not hydrolysed. Characterized by the production of rhamnolipid biosurfactant.

The type strain is JS^T (=KCTC 3924^T=DSM 16501^T=LMG 22864^T). Isolated from the traditional Korean food kimchi in Daejeon, South Korea.

Acknowledgements

This work was supported by grants from the Ministry of the Environment of the Republic of Korea; these grants are gratefully acknowledged.

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